JOURNAL OF BACTERIOLOGY, Feb. 1994, p. 917–922
0021-9193/94/$04.00+0

Vol. 176, No. 3

Cytoplasmic Axial Filaments in *Escherichia coli* Cells: Possible Function in the Mechanism of Chromosome Segregation and Cell Division

YOSHIO OKADA,[1,2]* MASAAKI WACHI,[3] AIKO HIRATA,[2] KOICHI SUZUKI,[2] KAZUO NAGAI,[3] AND MICHIO MATSUHASHI[4]

The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama-ken, 351-01 Japan,[1] Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, 113 Japan,[2] Department of Bioengineering, Tokyo Institute of Technology, Yokohama, Kanagawa-ken, 227 Japan,[3] and Department of Biological Science and Technology, Tokai University School of High Technology for Human Welfare, Numazu, Shizuoka-ken, 410-03 Japan[4]

Received 3 September 1993/Accepted 24 November 1993

Overproduction of CafA caused formation of chained cells and minicells. The *cafA* gene is located downstream from the *mre* region at 71 min on the *Escherichia coli* chromosome map and was previously called *orfF*. A long axial structure running through the chained cells, consisting of bundles of filaments assembled in a long hexagonal pillar several micrometers long and about 0.1 to 0.2 μm in diameter, was visible in both phase-contrast micrographs of the lysozyme-treated cells and electron micrographs of ultrathin sections. The CafA protein displays 34% amino acid similarity with the N terminus of the Ams protein of *E. coli*, which cross-reacts with antibody to a nonmuscle myosin heavy chain.

The *cafA* gene is located at 71 min on the *Escherichia coli* chromosome map, downstream from the shape determination genes *mreB*, *mreC*, and *mreD* (17, 18). It is flanked upstream by *orfE* and was previously called *orfF* (19). The functions of *orfE* and *cafA* have not been determined. We report here some significant effects of CafA overproduction on the cell morphology of *E. coli*, in particular the formation of singular intracellular structures which we call cytoplasmic axial filaments.

Construction of plasmids. Plasmids which carried a chromosomal fragment of *E. coli* encompassing either *cafA* (plasmid pGS2) or *orfE* located upstream from *cafA* (pGS1), or both *cafA* and *orfE* (pGS3) on high-copy-number plasmid pHSG398 (15), were isolated as shown in Fig. 1 and introduced into *E. coli* K-12 strain MV1184 [*ara* Δ(*lac-proAB*) *strA thi* (φ80 *lacZ*ΔM15) Δ(*srl-recA*)306::Tn*10*(Tet^r)/F′ *traD*36 *proAB lacI*^{q} *Z*ΔM15] (16). The transformants were grown in M9 medium supplemented with 1 mg of thiamine and 25 mg of chloramphenicol per liter, and cell shape was observed by phase-contrast microscopy (Fig. 2).

Formation of chained cells and minicells due to overproduction of CafA protein. Cells containing plasmids which carried the *cafA* gene (pGS2 and pGS3) formed cell chains containing round minicells (Fig. 1C and D) when the cells were grown in M9 medium containing 0.6 mM isopropyl-β-D-galactopyranoside (IPTG) to induce overproduction of a 51-kDa protein, CafA, the product of the *cafA* gene (Fig. 3A). No chained cells and minicells were formed and no 51-kDa protein was overproduced when the cells were grown in the absence of IPTG (data not shown). Cells containing the vector plasmid pHSG398 or plasmid pGS1, neither carrying the *cafA* gene, caused neither overproduction of CafA (Fig. 3A, lanes a, b, e, and f; Fig. 3B, lanes i, j, m, and n) nor formation of chained cells or minicells (Fig. 2A and B). The results were similar whether the cells were grown exponentially or grown slowly in the stationary phase.

Overproduced 51-kDa protein was purified from the cell extract by high-pressure liquid chromatography, and its N-terminal sequence was determined with a peptide sequencer. The sequence of the 10 N-terminal amino acid residues was Thr (or Gly)-Ala-Glu-Leu-Leu-Val-Asn (or Trp)-Val-Thr-Pro, in satisfactory agreement with the 11-amino-acid sequence deduced from the nucleotide sequence of the *cafA* gene, which is Met-Thr-Ala-Glu-Leu-Leu-Val-Asn-Val-Thr-Pro (19). The first methionine residue had been removed from the protein, and the amino group of the terminal threonine was not blocked (about 80% of the calculated amount of the N-terminal amino

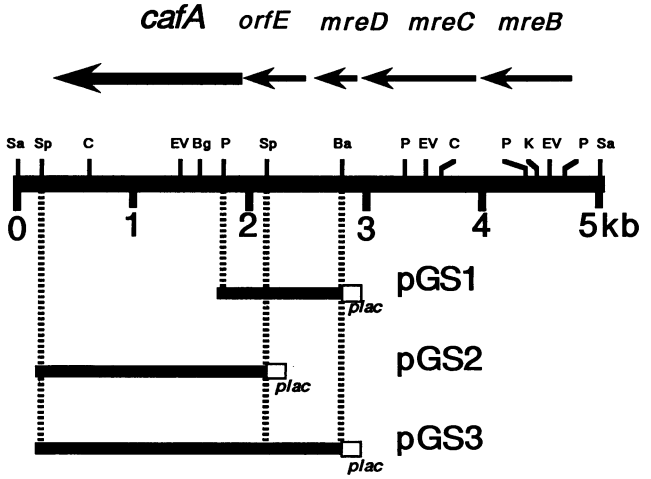


FIG. 1. Plasmid construction. Chromosomal fragments, each appropriately encompassing the gene *cafA*, the upstream flanking gene *orfE*, or both, were inserted into the multicloning site directly after the *lac* promoter (*plac*) of the high-copy-number plasmid pHSG398, which carries the chloramphenicol resistance gene and *lac* promoter (15), and the plasmids were used to transform *E. coli* MV1184. Abbreviations for restriction sites: Ba, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; EV, *Eco*RV; K, *Kpn*I; P, *Pst*I; Sa, *Sal*I; Sp, *Sph*I.

* Corresponding author. Mailing address: The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako, Saitama-ken, 351-01 Japan. Phone: (81.48)462-1111 ext. 5655. Fax: (81.48)462-4671.

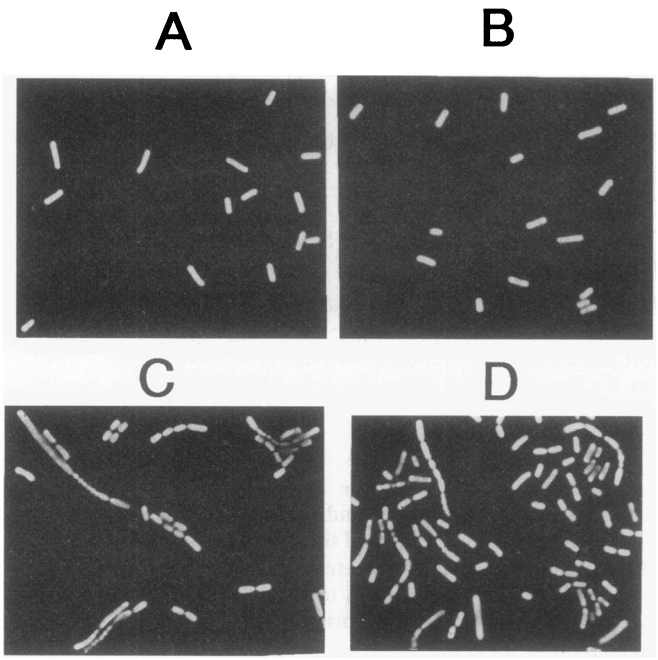


FIG. 2. Morphologies of cells overproducing CafA. Cells carrying the plasmids shown in Fig. 1 were grown at 30°C under shaking in M9 medium containing 1 mg of thiamine per liter, 25 mg of chloramphenicol per liter, and 0.6 mM IPTG for 40 h to stationary phase. Cells were suspended in saline, spotted on a thin film of 1% (wt/vol) agar in deionized water, covered with a coverglass, and examined under a dark-field phase-contrast microscope. (A) *E. coli* MV1184/pHSG398; (B) *E. coli* MV1184/pGS1; (C) *E. coli* MV1184/pGS2; (D) *E. coli* MV1184/pGS3. The bar represents 1 μm.

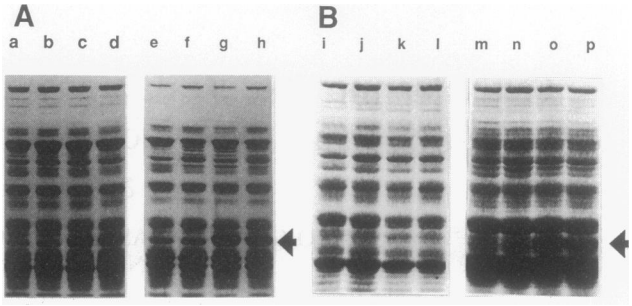


FIG. 3. Overproduction of CafA. Shown are Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoregrams of the cytoplasmic proteins. Cells and growth conditions were similar to those described for Fig. 2. (A) Cells grown in M9 broth supplemented with thiamine, chloramphenicol, and IPTG; (B) cells grown in Lennox broth (7) supplemented with 20 mg of thymine per liter, 25 mg of chloramphenicol per liter, and 0.6 mM IPTG. Growth temperature was 30°C. Lanes: a to d and i to l, logarithmically growing cells (A_{660} = 0.3); e to h and m to p, stationary-phase cells (A_{660} = 1.5 for lanes e to h and 3.0 for lanes m to p). *E. coli* cells used: lanes a, e, i, and m, MV1184/pHSG398; lanes b, f, j, and n, MV1184/pGS1; lanes c, g, k, and o, MV1184/pGS2; lanes d, h, l, and p, MV1184/pGS3. Arrows point to the 51-kDa protein. Overproduction of the 51-kDa protein was not observed in the absence of IPTG (not shown).

acid having been recovered as threonine). The 1,467-bp coding frame of *cafA* was expected to encode a protein with a molecular weight of 55,363, but the product migrated to the position of a 51-kDa protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Staining of the chromosome of the chained cells and minicells with 4′6-diamino-2-phenylindole revealed that most minicells and some normal-size cells in the chains had no chromosome (data not shown). The formation of anucleate cells and minicells suggests that overproduction of the CafA protein either enhances cell division beyond the normal rate or inhibits partition of chromosomes after replication or both.

Formation of giant spheroplasts and microscopic observation of cytoplasmic axial filaments. Upon treatment with lysozyme, the chained cells and minicells were converted into a unique large spheroplast, indicating that cell division may have been incomplete, the cytoplasm of the chained cells remaining connected through the constriction caused by uncompleted septation (Fig. 4). Moreover, an axial filamentous structure several micrometers in length and running throughout the inside of the spheroplast was visible under a phase-contrast microscope. This structure sometimes protruded from the spheroplast into the medium, suggesting a tough, elastic nature.

Electron microscopic observation of chained cells containing cytoplasmic axial filaments. Cells of *E. coli* MV1184/pGS2 were grown as described in the legend to Fig. 4 and then fixed by two different methods. In the conventional method, the cells were fixed in 3% glutaraldehyde for 2 h and then with 1% osmium tetroxide for 2 h and dehydrated with ethanol and acetone. In freeze-substituted fixation, which is especially suited for visualizing ultramicroscopic structures of eucaryotic cells (3, 6), cells collected by centrifugation were sandwiched between two copper meshes to form a thin layer of cells and plunged into liquid propane cooled with liquid nitrogen, causing the two meshes to cohere. The meshes were then peeled apart to form a thin aqueous layer containing the cells on the meshes. This material was transferred to 2% osmium tetroxide in anhydrous acetone at −80°C in a solid CO_2-acetone bath, maintained at this temperature for 48 h, at −35°C for 2 h, at 4°C for 2 h, and finally at room temperature for 2 h, and then washed with anhydrous acetone three times at room temperature. After fixation and dehydration, the samples were infiltrated with increasing concentrations of Spurr's resin (finally 100%) (14). The resin was polymerized in capsules at 50°C for 5 h and then at 70°C for 30 h. Thin sections were cut on a Reichert Ultracut N and stained at room temperature with uranyl acetate and then with lead citrate (12). Sections were viewed with a JEOL 200 CX electron microscope at 100 kV.

Electron microscopic observation of ultrathin sections of the cell chains revealed the presence of huge axial filamentous bundles running through the chained cells at the center of the cytoplasm (Fig. 5). These bundles were several micrometers long and 0.1 to 0.2 μm in diameter. Electron micrographs of lateral thin sections of the chained cells and minicells showed that the constrictions of the dividing cells were in fact unclosed, the space between the cell membranes and the filament bundles having been filled with cytoplasmic material (Fig. 5A and B). In single cells, bundles of filaments could be seen spanning the two cell poles at the cytoplasmic membranes (Fig. 5C). The structure appeared rigid and rather straight when the cells were fixed by a conventional glutaraldehyde method but appeared more elastic, like microtubules (eucaryotic cytoskeletal structures functioning in separation of the daughter chromosomes), when the cells were fixed by freeze substitution (Fig. 5D). Ultrathin sections perpendicular to the cell axis displayed sharp hexagonal cross sections of the axial structure at the center of the cell (Fig. 5B and E). These structures, which we call cytoplasmic axial filaments, were observed in almost every CafA-overproducing cell grown in the M9 culture.

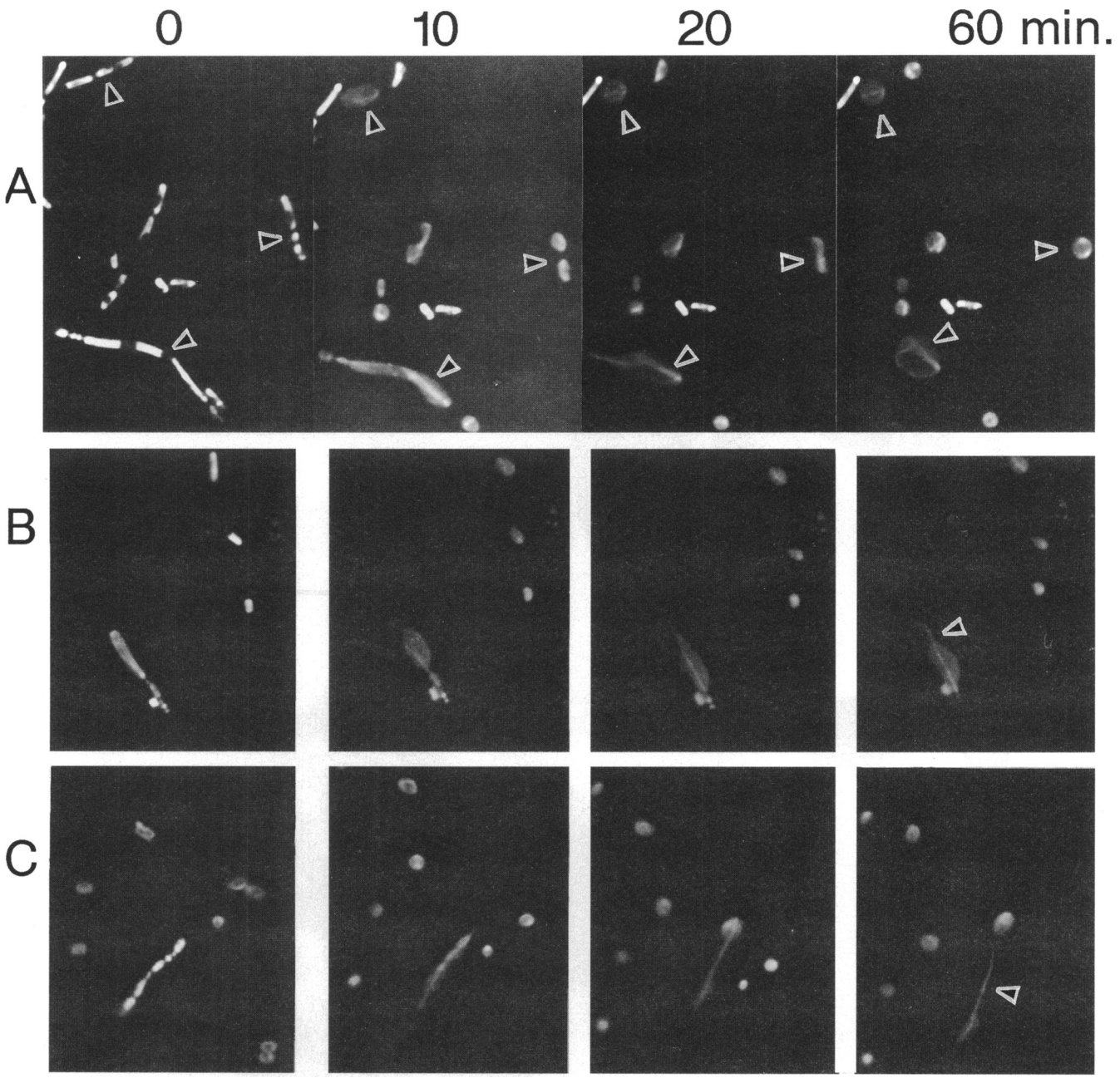


FIG. 4. Phase-contrast microscopic observation of unique giant spheroplasts formed from cell chains after treatment with lysozyme and appearance of cytoplasmic filament bundles. Cells of *E. coli* MV1184/pGS2 grown at 30°C for 40 h in Lennox broth (7) supplemented with thymine, chloramphenicol, and IPTG were suspended in spheroplasting buffer (pH 8.0) containing 100 mM Tris-HCl, 1 mM EDTA, and 15% sucrose and were spotted on a thin film of spheroplasting agar containing 1% agar and 50 μg of lysozyme per ml in spheroplasting buffer spread on a glass slide. The samples were covered with a coverglass, incubated at 20°C for the times indicated, and observed under a dark-field phase-contrast microscope. Panels A, B, and C are different views. Arrowheads show formation of unique spheroplasts from cell chains (A) and protrusion of filament structures (B and C). The bar represents 1 μm.

Do cytoplasmic axial filaments form a cytoskeletal structure involved in chromosome segregation and cell division? The cytoplasmic axial filaments could also be lysed or dispersed when cells overproducing CafA were inoculated into a rich, rapid growth medium such as Lennox broth (7) supplemented with 20 mg of thymine per liter with or without 0.6 mM IPTG. The filament bundles then disappeared from the electron micrographs, leaving small aggregated granules in the cytoplasm (electron micrographs not shown). This change in growth conditions caused a decrease in 51-kDa protein (Fig. 3B) and simultaneous disappearance of cell chains. New axial filament bundles appeared when the cells reached slow, stationary growth. Thus, turnover of the CafA protein and the axial filaments may occur. The cytoplasmic axial filaments may also be present in the rapidly growing cells, but only in an invisibly thin form. Quite probably, such a structure is also

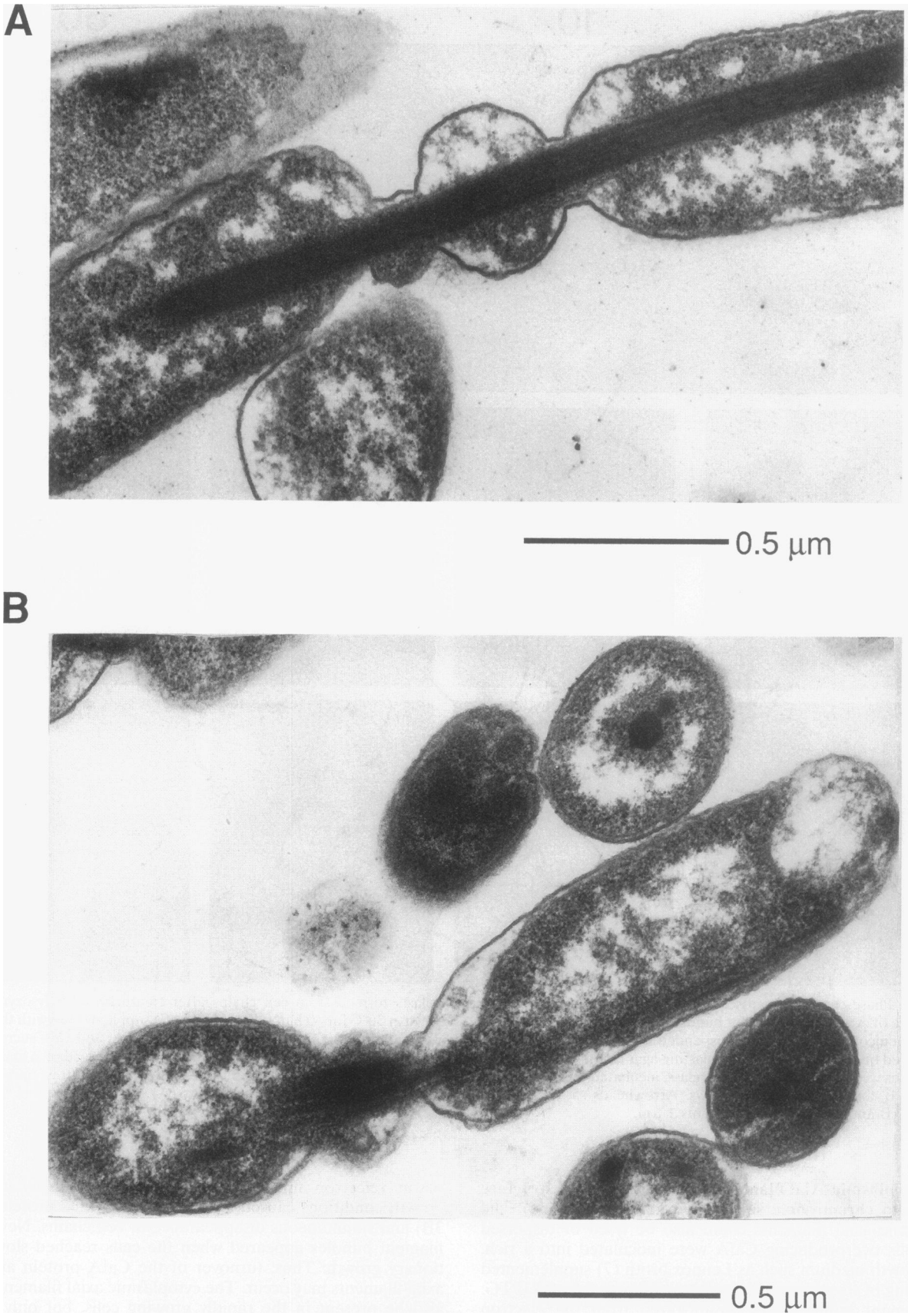
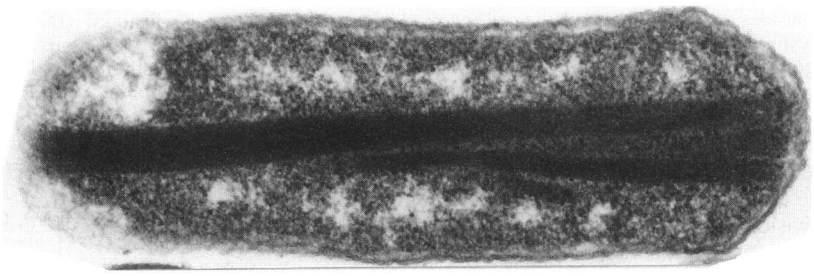


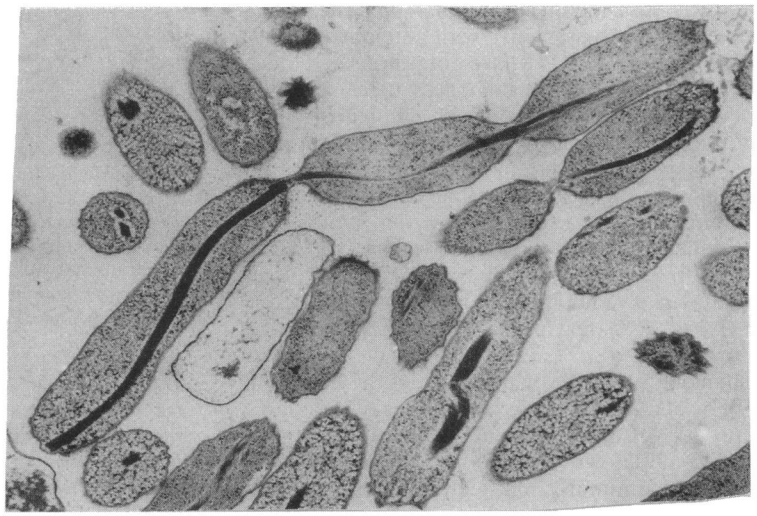
FIG. 5—*Continued.*

C



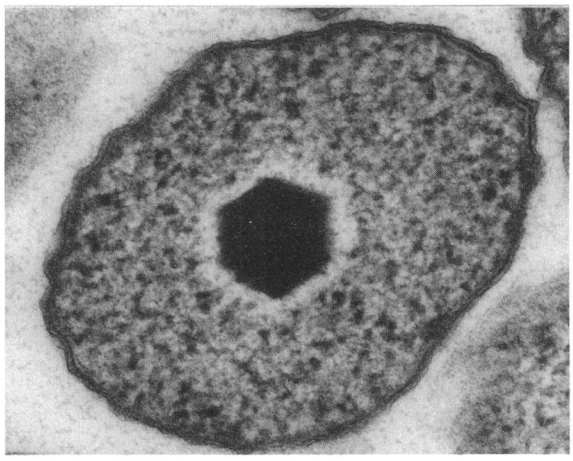
0.5 μm

D



1 μm

E



0.5 μm

FIG. 5—*Continued.*

FIG. 5. Electron micrographs of ultrathin sections of CafA-overproducing cells. Cells and growth conditions were similar to those described for Fig. 4. (A and B) Lateral sections of the cell chains containing minicells, showing bundles of straight cytoplasmic axial filaments (glutaraldehyde-osmium tetroxide fixation). Some cross sections are also visible. (C) Lateral section of a single cell containing multiple bundles of cytoplasmic axial filaments spanning the insides of the two poles of the cell (glutaraldehyde-osmium tetroxide fixation). (D) Lateral section of cell chains, showing rather flexible-looking cytoplasmic axial filament bundles (freeze-substituted fixation). Some cross sections are also visible. (E) Vertical section of a cell, showing a hexagonal cross section of the cytoplasmic axial filament bundle (freeze-substituted fixation).

present in normal cells and turns over during cell growth and division. If that is the case, then the structure in question may participate in cell division or chromosomal segregation. Formation of minicells is known to be caused by a defect in the normal regulation of cell division (13, 20). The possibility that the cytoplasmic axial filaments function as a cytoskeletal protein is also suggested by the fact that the amino acid sequence of the total CafA protein (19) displays similarity of 34% with the N-terminal portion of the 114-kDa protein encoded by the *ams* gene (1, 8). The *ams* gene, which is related to the stability of mRNAs, is identical with *hmp1* and *rne*, and the product of this gene is cross-reactive with antibody to the heavy chain of yeast myosin (1, 2). We have not yet purified the cytoplasmic axial filament bundles or compared their constituents with the CafA protein by amino acid sequencing or determination of cross-reactivity with antibody raised against the CafA protein. The possibility that the CafA protein is only one of the components of the cytoplasmic axial filament bundles, or merely regulates the formation of this structure, has not yet been ruled out. One possibility is that the dynamine-like 177-kDa protein encoded by the *mukB* gene (9), which is thought to be involved in the positioning of replicated chromosomes in *E. coli* (5, 9), may participate in the functioning of the CafA protein.

The upstream flanking *orfE* gene, which encodes the 22-kDa OrfE protein (19), displays a high degree of similarity with the 23-kDa protein product of *orfY* (reference 10 and GenBank accession number M96791), which is reported to be located upstream from *ams* with an intervening sequence, *orfX*. Similarity of the longer region *orfE-cafA* at 71 min on the *E. coli* chromosome map with *orfY*-(*orfX*)-*ams* at 24 min suggests closer biological functions of the corresponding genes. Although not yet established, the function of the Ams protein is thought to be cytokinetic, translocating RNA in the cell, rather than acting as an RNase itself (1, 8). Possibly the function of the CafA protein is also cytoskeletal, or cytokinetic, involved in chromosome segregation and cell division. However, we found no phenotypic changes in mutants defective in *cafA* (11, 17). Isolation of an appropriate double mutant of the relevant genes, such as a *cafA ams* mutant, may be necessary to determine the function of the *cafA* gene.

Similar axial structures which appeared when a mutant FtsA protein was overproduced have also been reported (4). These structures seem to be different in the size and location in the cell from those formed when the CafA protein was overproduced. Possibly there are multiple axial filamentous structures in the cell, which might function in compensating for the lack of each other.

This work was supported partially by grants-in-aid from the Ministry of Education, Science and Culture of Japan and Special Researcher's Basic Science Program of RIKEN.

REFERENCES

1. **Casarégola, S., A. Jacq, D. Laoudj, G. McGurk, S. Margarson, M. Tempête, V. Norris, and I. B. Holland.** 1992. Cloning and analysis of the entire *Escherichia coli ams* gene. *ams* is identical to *hmp1* and encodes a 114 kDa protein that migrates as a 180 kDa protein. J. Mol. Biol. **228:**30–40.
2. **Casarégola, S., V. Norris, M. Goldberg, and I. B. Holland.** 1990. Identification of a 180 kD protein in *Escherichia coli* related to a yeast heavy chain myosin. Mol. Microbiol. **4:**505–511.
3. **Escaig, J.** 1982. New instruments which facilitate rapid freezing at 83 K and 6 K. J. Microsc. **126:**221–229.
4. **Gayda, R. C., M. C. Henk, and D. Leong.** 1992. C-shaped cells caused by expression of an *ftsA* mutation in *Escherichia coli*. J. Bacteriol. **174:**5362–5370.
5. **Hiraga, S., T. Ogura, H. Niki, C. Ichinose, and H. Mori.** 1990. Positioning of replicated chromosomes in *Escherichia coli*. J. Bacteriol. **172:**31–39.
6. **Hobot, J. A., E. Carlemaln, W. Villiger, and E. Kellenberger.** 1984. Periplasmic gel: new concept resulting from the reinvestigation of bacterial cell envelope ultrastructure by new methods. J. Bacteriol. **160:**143–152.
7. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1:**190–206.
8. **McDowall, K. J., R. G. Hernandez, S. Lin-Chao, and S. N. Cohen.** 1993. The *ams-1* and *rne-3071* temperature-sensitive mutations in the *ams* gene are in close proximity to each other and cause substitutions within a domain that resembles a product of the *Escherichia coli mre* locus. J. Bacteriol. **175:**4245–4249.
9. **Niki, H., A. Jaffe, R. Imamura, T. Ogura, and S. Hiraga.** 1991. The new gene *mukB* codes for a 177 kD protein with coiled-coil domains involved in chromosome partitioning of *E. coli*. EMBO J. **10:**183–193.
10. **Oh, W., and T. J. Larson.** 1992. Physical locations of genes in the *rne*(*ams*)-*rpmF-plsX-fab* region of the *Escherichia coli* K-12 chromosome. J. Bacteriol. **174:**7873–7874.
11. **Okada, Y., M. Wachi, K. Nagai, and M. Matsuhashi.** 1992. Change of the quantity of penicillin-binding proteins and other cytoplasmic and membrane proteins by mutations of the cell shape-determination genes *mreB*, *mreC*, and *mreD* of *Escherichia coli*. J. Gen. Appl. Microbiol. **38:**157–163.
12. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17:**208–212.
13. **Rothfield, L. I., P. DeBoer, and W. R. Cook.** 1990. Localization of septation sites. Res. Microbiol. **141:**57–63.
14. **Spurr, A. R.** 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26:**31–43.
15. **Takeshita, S., M. Sato, M. Toba, W. Masahashi, and T. Hishimoto-Gotoh.** 1987. High-copy-number and low-copy-number plasmid vectors for lacZα-complementation and chloramphenicol- or kanamycin-resistance selection. Gene **61:**63–74.
16. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153:**3–11.
17. **Wachi, M., M. Doi, Y. Okada, and M. Matsuhashi.** 1989. New *mre* genes *mreC* and *mreD*, responsible for formation of the rod shape of *Escherichia coli* cells. J. Bacteriol. **171:**6511–6516.
18. **Wachi, M., M. Doi, S. Tamaki, W. Park, S. Nakajima-Iijima, and M. Matsuhashi.** 1987. Mutant isolation and molecular cloning of *mre* genes, which determine cell shape, sensitivity to mecillinam, and amount of penicillin-binding proteins in *Escherichia coli*. J. Bacteriol. **169:**4935–4940.
19. **Wachi, M., M. Doi, T. Ueda, M. Ueki, K. Tsuritani, K. Nagai, and M. Matsuhashi.** 1991. Sequence of the downstream flanking region of the shape-determining genes *mreBCD* of *Escherichia coli*. Gene **106:**135–136.
20. **Ward, J. E., Jr., and J. Lutkenhaus.** 1985. Overproduction of FtsZ induces minicell formation in *E. coli*. Cell **42:**941–949.